DESCRIPTION

ADIPONECTIN RECEPTOR AND GENE ENCODING THE SAME

RELATED APPLICATIONS

This application is a continuation of PCT/JP03/07515 entitled "Adiponectin Receptor and Gene Encoding the Same", filed June 12, 2003, which claims priority to Japanese Application No. 2002-383738 filed December 29, 2002. Each of the aforementioned applications are hereby incorporated herein by reference in their entirety.

TECHNICAL FIELD

The present invention relates to a novel protein having adiponectin-binding ability, a gene encoding the protein, a recombinant vector containing the gene, a transformant containing the recombinant vector, and an antibody to the protein and a fragment of the antibody. Moreover, the present invention relates to a screening method and a screening kit for a ligand, agonist or antagonist to an adiponectin receptor.

BACKGROUND ART

Obesity is defined as an increased mass of adipose tissue, and is associated with a higher risk of cardiovascular and metabolic disorders such as diabetes, hyperlipidemia and coronary heart disease (Reaven, G.M., *Diabetologia* 38, 3-13 (1995); Spiegelman, B.M. et al, *Cell* 87, 377-389 (1996)). Impaired glucose and lipid metabolism, a hallmark of obesity and type 2 diabetes, causes increased lipid storage in insulin target tissues such as muscle and liver, thereby leading to insulin resistance (Ruderman, N.B. et al, *Am. J. Physiol.* 276, E1-E18 (1999); Shulman, G.I., *J.*

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Clin. Invest. 106, 171-176 (2000)). The adipose tissue
itself serves as the site of triglyceride (TG) storage and
free fatty acids (FFA)/glycerol release in response to
changing energy demands (Spiegelman, B.M. and Flier, J.S.,
Cell 87, 377-389 (1996)). Adipose tissue also participates
in the regulation of various types of energy homeostasis as
an important endocrine organ that secretes a number of
biologically active substances called "adipokines"
(Matsuzawa, Y. et al, *Ann. NY Acad. Sci.* 892, 146-154
(1999)) such as FFA (Shulman, G.I., *J. Clin. Invest.* 106,
171-176 (2000)), adipsin (White, R.T. et al, *J. Biol. Chem.*
267, 9210-9213 (1992)), leptin (Friedman, J.M., *Nature* 404,
632-634 (2000)), plasminogen activator inhibitor-1 (PAI-1)
(Shimomura, I. et al, *Nat. Med.* 2, 800-803 (1996)), resistin
(Steppan, C.M. et al, *Nature* 409, 307-312 (2001)) and tumor
necrosis factor-α (TNF-α) (Hotamisligil, G.S., *J. Intern.
Med.* 245, 621-625 (1999)).

Adiponectin or Acrp30 (Hu, E., Liang, P. et al, *J. Biol
Chem.* 271, 10697-10703 (1996) and others) is an adipocyte-
derived hormone with multiple biological functions. It has
been reported that obesity, type 2 diabetes and coronary
heart disease are associated with decreased plasma
adiponectin levels, and that adiponectin may have putative
anti-atherogenic properties *in vitro* (Ouchi, N. et al,

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Circulation 103, 1057-1063 (2001); Yokota, T. et al, *Blood* 96, 1723-1732 (2000)). Also, it has been reported that an acute increase in circulating levels of Acrp30 lowers hepatic glucose production (Berg, A.H. et al, *Nat. Med.* 7, 947-953 (2001); Combs, T.P. et al, *J. Clin. Invest.* 108, 1875-1881 (2001)). Also, it has been reported that globular Acrp30 increases fatty acid oxidation in muscle, and causes weight loss in mice (Fruebis, J. et al, *Proc. Natl. Acad. Sci. USA* 98, 2005-2010 (2001)). Also, it has been reported that treatment with adiponectin consisting solely of the globular domain (globular adiponectin or gAd) increases fatty acid oxidation in muscle, thereby ameliorating insulin resistance in lipoatrophic mice and obese mice, while treatment with full-length adiponectin also ameliorates though less than with gAd (Yamauchi, T. et al, *Nat. Med.* 7, 941-946 (2001)).

Recently it has been reported that adiponectin acutely activates AMP kinase (AMPK) in skeletal muscle, thus stimulating fatty acid oxidation and glucose uptake (Yamauchi, T. et al, *Nat. Med.* 8, 1288-1295 (2002)), and that adiponectin chronically activates PPARα, resulting in increased fatty acid oxidation but reduced tissue TG content in the muscles, with these effects being greater with gAd than with full-length adiponectin (Yamauchi, T. et al, *J.*

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Biol. Chem. 278, 2461-2468 (2002)). Interestingly, in the liver full-length adiponectin alone acutely activates AMPK, causing a reduction in gluconeogenesis-associated molecules and stimulating fatty-acid oxidation, and moreover full-length adiponectin alone chronically activates AMPK, stimulating fatty-acid oxidation and reducing tissue TG levels in the liver. All these changes serve to enhance insulin sensitivity *in vivo* (Yamauchi, T. et al, *Nat. Med.* 8, 1288-1295 (2002); Yamauchi, T. et al, *J. Biol. Chem.* 278, 2461-2468 (2002)).

These effects of adiponectin are believed to be mediated by receptors on the cell surface, but adiponectin receptors have not been identified, and it is unknown whether the adiponectin receptors in the skeletal muscle and liver differ either structurally or functionally. The inventors identified a gene encoding adiponectin receptors, and discovered from a homology search that yeast YOL002c gene is a homologue (Karpichev, I.V. et al, *Journal of Biological Chemistry* 277, 19609-19617 (2002)). YOL002c encodes a seven transmembrane protein that plays a key role in the metabolic pathways of lipids, such as fatty acid oxidation (Karpichev, I.V. et al, *Journal of Biological Chemistry* 277, 19609-19617 (2002)).

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DISCLOSURE OF THE INVENTION

First, it is an object of the present invention to provide a novel protein having adiponectin-binding ability, a gene encoding the protein, a recombinant vector containing the gene, a transformant containing the recombinant vector, and an antibody to the protein and a fragment of the antibody.

Second, it is an object of the present invention to provide a screening method and screening kit for a ligand, agonist or antagonist to an adiponectin receptor.

In order to achieve these objects, the present invention provides the following protein, gene, recombinant vector, transformant and antibody, as well as a screening method and screening kit for a ligand, agonist or antagonist to an adiponectin receptor.

(1)   A protein as set forth in (a) or (b) below:

(a)   a protein comprising an amino acid sequence according to Seq. No. 2, 4, 6 or 8; or

(b)   a protein comprising an amino acid sequence according to Seq. No. 2, 4, 6 or 8 with one or more amino acids deleted, replaced or added, and having adiponectin binding ability.

(2)   A gene encoding the protein according to the abovementioned (1).

(3)   The gene according to the abovementioned (2),
comprising DNA as set forth in (c) or (d) below:

    (c)   DNA comprising a base sequence according to Seq.
No. 1, 3, 5 or 7; or

    (d)   DNA which hybridizes under stringent conditions
with DNA complementary to DNA comprising a base sequences
according to Seq. No. 1, 3, 5 or 7, and which encodes a
protein having adiponectin binding ability.

(4)   A recombinant vector containing the gene according to
the abovementioned (2) or (3).

(5)   A transformant containing the recombinant vector
according to the abovementioned (4).

(6)   An antibody or a fragment thereof capable of reacting
with the protein according to the abovementioned (1).

(7)   A screening method for a ligand, agonist or antagonist
to an adiponectin receptor, comprising a step of bringing a
test substance into contact with the protein according to
the abovementioned (1).

(8)   A screening kit for a ligand, agonist or antagonist to
an adiponectin receptor, comprising the protein according to
the abovementioned (1), the DNA according to the
abovementioned (2) or (3), and the recombinant vector
according to abovementioned (4) or the transformant
according to abovementioned (5).

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1(a) shows the binding of adiponectin to C2C12 myocytes, Fig. 1(b) shows the binding of adiponectin to hepatocytes, Figs. 1(c) through 1(e) show the results of FACS analysis, Fig. 1(f) shows a schematic view of the structure of AdipoR1 and AdipoR2 genes transcripts, Fig. 1(g) shows the results of northern blot analysis for various mouse tissues, and Fig. 1(h) shows the results of northern blot analysis for various human tissues;

Fig. 2(a) shows a schematic view of the structures of AdipoR1 and AdipoR2, Fig. 2(b) shows the results of immunoblotting with anti-FLAG antibodies of cell lysates from 293T cells transfected with AdopR1 and AdipoR2 having the epitope tag FLAG, Fig. 2(c) shows the intracellular locations of AdipoR1 or AdipoR2 in 293T cells transfected with AdipoR1 and AdipoR2 having epitope flags introduced at the N or C end, and Figs. 2(d) and 2(e) show the predicted structural models of Adipo R1 and AdipoR2, respectively;

Fig. 3 (a) shows the binding isotherm of [^{125}I] globular Adipo (gAd) binding to 293T cells transfected with AdipoR1 or AdipoR2, Fig. 3(b) shows the binding isotherm of [^{125}I] full-length Adipo (Ad) binding to the 293T cells, Fig. 3(c) shows the results when cell lysates of 293T cells

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transfected with AdipoR1 or AdipoR2 having the epitope tag
FLAG or HA are immunoprecipitated with an anti-FLAG or anti-
HA antibody, and then immunoblotted with the anti-FLAG or
anti-HA antibody, Fig. 3(d) shows changes in $[Ca^{2+}]i$ when
mouse AdipoR1-expressing cells or BLT1-expressing cells were
challenged with globular Adipo, full-length Adipo, LTB4 or
ATP, Fig. 3(e) shows the results for accumulation of cAMP or
cGMP in HEK-29 cells treated with or without forskolin, and
Fig. 3(f) shows PPARα ligand activity in 293T cells
transfected with AdipoR1 that are incubated with globular
Adipo or full-length Adipo;

Fig. 4(a) shows mouse AdipoR1 mRNA levels, Fig. 4(b)
shows mouse AdipoR2 mRNA levels, Figs. 4(c) and 4(d) show
binding isotherms of [^{125}I] globular Adipo (gAd) or full-
length Adipo (Ad) binding to C2C12 myocytes transfected with
mouse AdipoR1 or AdipoR2, Fig. 4(e) shows PPARα ligand
activity in C2C12 myocytes transfected with mouse AdipoR1 or
Adipo R2 which were infected with an adenovirus containing
LacZ or DN-α2AMPK and treated for 7 hours with globular
Adipo or full-length Adipo, and Fig. 4(f) shows in vitro
fatty acid oxidation in the C2C12 myocytes;

Fig. 5(a) shows mouse AdipoR1 mRNA levels in C2C12
myocytes transfected with siRNA or mock, Fig. 5(b) shows

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mouse AdipoR2 mRNA levels in the C2C12 myocytes, Fig. 5(c)

shows the results of a competitive radioligand binding assay

in which [^{125}I] globular Adipo binding to cells transfected

with siRNA dublex is displaced by increasing concentrations

of unlabeled globular Adipo, Fig. 5(d) shows the results of

a competitive radioligand binding assay in which [^{125}I] full-

length Adipo binding to cells transfected with siRNA duplex

is displaced by increasing concentrations of unlabeled full-

length Adipo, Fig. 5(e) shows the binding isotherm of [^{125}I]

globular Adipo binding to C2C12 myocytes transfected with

siRNA duplex, Fig. 5(f) shows the binding isotherm of [^{125}I]

full-length Adipo binding to C2C12 myocytes transfected with

siRNA duplex, Fig. 5(g) shows PPARα ligand activity in

C2C12 myocytes transfected with siRNA duplex which are

incubated for 7 hours with globular Adipo, full-length Adipo

or Wy-14,643, Fig. 5(h) shows in vitro fatty acid oxidation

in C2C12 myocytes transfected with siRNA duplex which are

incubated for 7 hours with globular Adipo, full-length Adipo

or Wy-14,643, and Fig. 5(i) shows glucose uptake in C2C12

myocytes transfected with siRNA duplex which are incubated

for 7 hours with globular Adipo, full-length Adipo or

insulin;

Fig. 6(a) shows specific binding of gAd to hepatocytes,

Fig. 6(b) shows specific binding of Ad to hepatocytes, Fig.

6(c) shows expression levels of human AdipoR1 mRNA in HAEC,

Fig. 6(d) shows expression levels of human AdipoR2 mRNA in

HAEC, Fig. 6(e) shows specific binding of gAd to HAEC, and

Fig. 6(f) shows specific binding of Ad to HAEC;

Fig. 7(a) shows the binding isotherm of [^{125}I] globular

Adipo binding to C2C12 cells transfected with siRNA duplex,

Fig. 7(b) shows the binding isotherm of [^{125}I] full-length

Adipo binding to C2C12 myocytes transfected with siRNA

duplex, Fig. 7(c) shows the results of Scatchard plot

analysis based on the results shown in Fig. 7(a), and Fig.

7(d) shows the results of Scatchard plot analysis based on

the results shown in Fig. 7(b); and

Fig. 8(a) shows the results when C2C12 cells

transfected or not transfected with AdipoR1 are incubated

for 10 minutes with 0.1 µg/mL or 1 µg/mL of gAd and a lysate

of the cells was then reacted with anti-phosphorylated AMPK

antibodies, Fig. 8(b) shows the results when C2C12 cells

transfected or not transfected with AdipoR1 are incubated

for 10 minutes with 0.1 µg/mL or 1 µg/mL of gAd and a lysate

of the cells is then reacted with anti-phosphorylated ACC

antibodies, Fig. 8(c) shows the results when C2C12 cells

transfected or not transfected with AdipoR1 are incubated

for 10 minutes with 0.1 µg/mL or 1 µg/mL of gAd and a lysate

of the cells is then reacted with anti-phosphorylated p38

MAPK antibodies, Fig. 8(d) shows the results when C2C12 cells transfected or not transfected with AdipoR1 are incubated for 10 minutes with 0.1 µg/mL or 1 µg/mL of gAd and a lysate of the cells is then reacted with anti-phosphorylated MAPK antibodies, Fig. 8(e) shows the results when hepatocytes transfected or not transfected with AdipoR1 were incubated for 10 minutes with 0.1 µg/mL or 1 µg/mL of gAd and a lysate of the cells was then reacted with anti-phosphorylated AMPK antibodies, Fig. 8(f) shows the results when hepatocytes transfected or not transfected with AdipoR1 are incubated for 10 minutes with 0.1 µg/mL or 1 µg/mL of gAd and a lysate of the cells is then reacted with anti-phosphorylated ACC antibodies, Fig. 8(g) shows fatty acid oxidation of C2C12 cells transfected or not transfected with AdipoR1 in the presence of dominant negative AMP kinase (DN-AMPK) or p38 MAPK-specific inhibitor SB203580, and Fig. 8(h) shows glucose intake of C2C12 cells transfected or not transfected with AdipoR1 in the presence of dominant negative AMP kinase (DN-AMPK) or p38 MAPK-specific inhibitor SB203580.

BEST MODE FOR CARRYING OUT THE INVENTION

A protein of the present invention is a protein as set forth in (a) or (b) below:

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(a) a protein comprising an amino acid sequences according to Seq. No. 2, 4, 6 or 8 (hereunder "protein (a)");

(b) a protein comprising an amino acid sequences according to Seq. No. 2, 4, 6 or 8 with one or more amino acids deleted, replaced or added, and having adiponectin binding ability (hereunder "protein (b)").

Protein (a) or (b) has adiponectin binding ability. "Adiponectin binding ability" signifies the ability to bind with adiponectin, and preferably the ability to bind specifically with adiponectin (that is, the adiponectin binding ability of an adiponectin receptor). "Adiponectin binding ability" includes binding ability with respect to either full-length adiponectin or adiponectin consisting only of the globular domain. Proteins having adiponectin binding ability include proteins that can bind only to full-length adiponectin, proteins that can bind only to adiponectin consisting only of the globular domain, proteins that can bind to both full-length adiponectin and adiponectin consisting only of the globular domain, and proteins which bind preferentially to either full-length adiponectin or adiponectin consisting only of the globular domain.

The "globular domain" is the filament-like structural domain at the C end of adiponectin, having a length of a little over 100 amino acids. Complement C1q and the like have domains highly homologous to the globular domain. Functionally, the globular domain of adiponectin acts strongly on skeletal muscle and the like, combusting fatty acids and inhibiting the storage of lipids, and it exhibits the same level of activity as full-length adiponectin at a concentration as low as one-several tenth of that of the full-length adiponectin.

Of proteins (a), those according to Seq. No. 2 or 4 are human adiponectin receptors, while those according to Seq. No. 6 or 8 are mouse adiponectin receptors. The proteins according to Seq. Nos. 2 and 6 are the same kind of adiponectin receptor (AdipoR1), while those according to Seq. Nos. 4 and 8 are a kind of adiponectin receptor (AdipoR2) different from AdipoR1. AdipoR1 is thought to be a receptor with relative selectivity for adiponectin consisting solely of the globular domain rather than full-length adiponectin, while AdipoR2 is thought to be a receptor with relative selectivity for full-length adiponectin rather than adiponectin consisting solely of the globular domain.

The degree of homology between human (Seq. No. 2) and mouse (Seq. No. 6) AdipoR1 at the amino acid level is 96.8%.

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The degree of homology between human (Seq. No. 4) and mouse (Seq. No. 8) AdipoR2 at the amino acid level is 95.2%. The structures of AdipoR1 and AdipoR2 are similar, and the homology between AdipoR1 (Seq. No. 6) and AdipoR2 (Seq. No. 8) in mice is 66.7%.

In vivo, AdipoR1 is expressed in most tissues, with especially high expression in skeletal muscle, while AdipoR2 is expressed strongly in the liver. AdipoR1 and AdipoR2 are thought to form both homo- and hetero-multimers. Both AdipoR1 and AdipoR2 bind with both full-length adiponectin and adiponectin consisting solely of the globular domain, mediating the stimulating effects of these adiponectins on PPARα (Peroxisome proliferator-activated receptor α) ligand activity and fatty acid oxidation. For example, AdipoR1 is thought to mediate the increases in PPARα ligand activity, fatty acid oxidation and glucose uptake by adiponectin consisting only of the globular domain in myocytes. Similarly, AdipoR2 is thought to partially mediate the increases in PPARα ligand activity and fatty acid oxidation by full-length adiponectin in hepatocytes and myocytes.

AdipoR1 and AdipoR2 Are both thought to contain seven transmembrane domains. In the amino acid sequence according to Seq. No. 2 (human AdipoR1), the amino acid sequences 136-

158, 172-194, 207-228, 234-255, 267-289, 299-321 and 336-358 are though to correspond to transmembrane domains. In the amino acid sequence according to Seq. No. 4 (human AdipoR2), the amino acids sequences 60-82, 96-118, 130-152, 158-179, 192-214, 222-244 and 260-282 are though to correspond to transmembrane domains. In the amino acid sequence according to Seq. No. 6 (mouse AdipoR1), the amino acid sequences 136-158, 172-194, 207-228, 234-255, 267-289, 299-321 and 336-358 are though to correspond to transmembrane domains. Finally, in the amino acid sequence according to Seq. No. 8 (mouse AdipoR2), the amino acid sequences 72-94, 108-130, 142-164, 170-191, 204-226, 234-256 and 272-294 are though to correspond to transmembrane domains.

There are no particular limits on the number of amino acids deleted, replaced or added in the amino acid sequences according to Seq. Nos. 2, 4, 6 and 8 as long as adiponectin binding ability is retained. The number may be one or more than one or preferably one or a few, or specifically in the range of normally 1-100 or preferably 1-50 or more preferably 1-10. The amino acid sequences of protein (b) should normally have 60% or more, or preferably 80% or more, or more preferably 90% or more homology with the amino acid sequence of protein (a).

There are no particular limits on the locations of the amino acids deleted, replaced or added in the amino acid sequences according to Seq. No. 2, 4, 6 or 8, as long as adiponectin binding ability is retained.

Protein (b) includes proteins wherein deletions, replacements, additions and other mutations have been artificially introduced into a protein (a), as well as proteins naturally occurring with introduced deletions, replacements, additions and other mutations and such proteins with deletions, replacements, additions and other mutations artificially introduced. Examples of proteins naturally occurring with introduced deletions, replacements, additions and other mutations include proteins derived from humans and other mammals such as monkeys, cows, sheep, goats, horses, pigs, rabbits, dogs, cats, mice, rats and the like (including proteins which may occur due to polymorphisms in such mammals).

Proteins (a) and (b) include both proteins with added sugar chains and proteins without added sugar chains. The types, locations and the like of the sugar chains added to the proteins will differ depending on the type of host cells used in manufacturing the protein, but proteins with added sugar chains include proteins obtained using any kind of

host cells. Proteins (a) and (b) also include

pharmacologically allowable salts thereof.

A gene encoding protein (a) or (b) can be obtained for

example by using mRNA extracted from the skeletal muscle,

livers, hearts, macrophages, blood vessels, brains, kidneys,

lungs, placentas, spleens, testes, peripheral blood, thymus

glands, intestines or other tissues of humans, mice or other

mammals to build a cDNA library, and then using probes

synthesized based on the base sequences according to Seq. No.

1, 3, 5 or 7 to screen the cDNA library for clones

containing the target DNA. The steps involved in preparing

a cDNA library and screening for clones containing the

target DNA are explained below.

[Preparing a cDNA library]

To prepare a cDNA library, total RNA is first obtained

from the skeletal muscle, livers, hearts, macrophages, blood

vessels, brains, kidneys, lungs, placentas, spleens, testes,

peripheral blood, thymus glands, intestines or other tissues

of humans, mice or other mammals for example, and

poly(A+)RNA (mRNA) is then obtained by the batch method,

affinity column method or the like using oligo(dT)-cellulose,

poly(U)-sepharose or the like. The poly(A+)RNA (mRNA) in

this case may also be fractionated by sucrose density

gradient centrifugation or the like. Taking the resulting

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mRNA as the template, single-stranded cDNA is then synthesized using oligo(dT) primer and reverse transcriptase, and double-stranded cDNA is synthesized from the single-stranded cDNA. The resulting double-stranded cDNA is incorporated into a suitable cloning vector to prepare a recombinant vector which is used to transform *E. coli* or other host cells, and a cDNA library is obtained by selection of transformants using tetracycline resistance or ampicillin resistance as the indicator. The cloning vector for preparing the cDNA library may be any vector that can replicate independently in the host cells, and for example phage vectors, plasmid vectors and the like can be used. *E. coli* or the like can be used as the host cells.

Transformation of the *E. coli* or other host cells can be accomplished by a method such as adding the recombinant vector to competent cells prepared with calcium chloride, magnesium chloride or rubidium chloride. When a plasmid is used as the vector, a resistance gene for a drug such as tetracycline or ampicillin is included.

A commercial kit such as the SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (Gibco BRL) or the ZAP-cDNA Synthesis Kit (Stratagene) can be used in preparing the cDNA library.

[Screening for clones containing the target DNA]

To screen the cDNA library for clones containing the target DNA, primer is synthesized based on the base sequence according to Seq. No. 1, 3, 5 or 7, and used in a polymerase chain reaction (PCR) to obtain PCR amplified fragments. The PCR amplified fragments may also be sub-cloned using a suitable plasmid vector. There are no particular limits on the primer set used in PCR, which can be designed based on the base sequence according to Seq. Nos. 1, 3, 5 or 7.

The target DNA is obtained by colony hybridization or plaque hybridization of the cDNA library, with the PCR amplified fragments as the probe. The PCR amplified fragments may be labeled with an isotope (such as ^{32}P or ^{35}S), biotin, digoxigenin, alkaliphosphotase or the like for use as the probe. Clones containing the target DNA are obtained by an expression screening method such as immunoscreening using antibodies.

The resulting DNA can be incorporated into a vector by ordinary methods, either as the original DNA fragments or after cleavage with a suitable restriction enzyme or the like, and the base sequence can be determined by an ordinarily used method of base sequence analysis, such as the Maxam-Gilbert chemical modification method or the dideoxynucleotide chain termination method. A base sequence

analyzing device such as the 373A DNA Sequencer (Perkin Elmer) is normally used in analyzing the base sequence.

A gene encoding protein (a) or (b) comprises an open reading frame encoding protein (a) or (b) and the termination codon located at the 3' end thereof. Moreover, a gene encoding protein (a) or (b) can comprise an untranslated region (UTR) at the 5' and/or 3' ends of the open reading frame.

Examples of the gene encoding protein (a) include a gene comprising DNA comprising a base sequence according to Seq. No. 1, 3, 5 or 7. Of the base sequence according to Seq. No. 1, bases 1-1125 constitute the open reading frame which encodes the protein according to Seq. No. 2, while the translation initiation codon is located at bases 1-3 of the base sequence according to Seq. No. 1, and the termination codon at bases 1126-1128 of the sequence. Of the base sequence according to Seq. No. 3, bases 1-897 constitute the open reading frame which encodes the protein according to Seq. No. 4, while the translation initiation codon is located at bases 1-3 of the base sequence according to Seq. No. 3, and the termination codon at bases 898-900. Of the base sequence according to Seq. No. 5, bases 1-1125 constitute the open reading frame which encodes the protein according to Seq. No. 6, while the translation initiation

codon is located at bases 1-3 of the base sequence according to Seq. No. 5, and the termination codon at bases 1126-1128. Of the base sequence according to Seq. No. 7, bases 1-933 constitute the open reading frame which encodes the protein according to Seq. No. 8, while the translation initiation codon is located at bases 1-3 of the base sequence according to Seq. No. 7, and the termination codon at bases 934-936.

There are no particular limits on the base sequence of the gene encoding protein (a) so long as it encodes protein (a), and the base sequence of the open reading frame is not restricted to a base sequence according to Seq. No. 1, 3, 5 or 7. The gene encoding protein (a) can also be obtained by chemical synthesis according to its base sequence. Chemical synthesis of DNA can be accomplished using a commercial DNA synthesizer, such as a DNA synthesizer using the thiophosphite method (Shimadzu Corporation) or the phosphoramidite method (Perkin Elmer).

An example of a gene encoding protein (b) is a gene comprising DNA which hybridizes under stringent conditions with DNA complementary to DNA comprising a base sequence according to Seq. No. 1, 3, 5 or 7, and which encodes a protein having adiponectin binding ability.

An example of "stringent conditions" would be for example 42°C, 2 X SSC and 0.1% SDS, or preferably 65°C, 0.1 X SSC and 0.1% SDS.

A specific example of DNA which hybridizes under stringent conditions with DNA complementary to DNA comprising a base sequence according to Seq. No. 1, 3, 5 or 7 is DNA having 60% or more or preferably 80% or more or more preferably 90% or more homology with a base sequence according to Seq. No. 1, 3, 5 or 7.

The gne encoding protein (b) can be obtained for example by artificially introducing mutations into DNA comprising a base sequence according to Seq. No. 1, 3, 5 or 7, using a well-known method such as site-directed mutagenesis. Mutations can be introduced using a mutagenesis kit, such as a Mutant-K (Takara), Mutant-G (Takara) or Takara LA PCR in vitro Mutagenesis series kit. DNA whose base sequence has already been determined can be obtained by chemical synthesis.

Protein (a) or (b) can be manufactured according to the following steps by expressing the gene encoding each protein in host cells.

[Preparation of recombinant vectors and transformants]

To prepare a recombinant vector, a DNA fragment of a suitable length is prepared which comprises a region

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encoding the target protein. The DNA is also prepared wherein bases of the base sequence of the protein-encoding region are replaced so that the codons are optimized for expression in the host cells.

A recombinant vector is prepared by inserting this DNA fragment downstream from the promoter of a suitable expression vector, and a transformant capable of producing the target protein is obtained by introducing the recombinant vector into suitable host cells. The DNA fragment needs to be incorporated into the vector so that its functions are expressed, and the vector may contain in addition to the promoter enhancers or other cis-elements, splicing signals, poly(A) addition signals, selection markers (such as dihydrofolate reductase gene, ampicillin resistance gene or neomycin resistance gene), ribosome binding sequences (SD sequences) or the like.

There are no particular limits on the expression vector as long as it can replicate independently in the host cells, and for example plasmid vectors, phage vectors, virus vectors and the like can be used. Examples of plasmid vectors include *E. coli*-derived plasmids (such as pRSET, pBR322, pBR325, pUC118, pUC119, pUC18 and pUC19), *Bacillus subtilis*-derived plasmids (such as pUB110 and pTP5) and yeast-derived plasmids (such as YEp13, YEp24 and Ycp50).

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Examples of phage vectors include λ-phages (such as Charon4A, Charon21A, EMBL3, EMBL4, λgt10, λgt11 and λZAP), and examples of virus vectors include retroviruses, vaccinia virus and other animal viruses and baculoviruses and other insect viruses.

As long as the host cells can express the target DNA, they can be prokaryotic cells, yeasts, animal cells, insects cells, plant cells or the like. An animal body, plant body, silkworm body or the like can also be used.

When bacteria are used as the host cells, they can be for example *Escherichia coli* or other Escherichia, *Bacillus subtilis* or other Bacillus, *Pseudomonas putida* or other Pseudomonas, or *Rhizobium meliloti* or other Rhizobium bacteria. Specifically, *Escherichia coli* XL1-Blue, *Escherichia coli* XL2-Blue, *Escherichia coli* DH1, *Escherichia coli* K12, *Escherichia coli* JM109, *Escherichia coli* HB101 and other *Escherichia coli* and *Bacillus subtilis* MI 114, *Bacillus subtilis* 207-21 and other *Bacillus subtilis* can be used as the host cells. There are no particular limits on the promoter in this case as long as it is capable of expression in *E. coli* or other bacteria, and for example a trp promoter, lac promoter, P_L promoter, P_R promoter or other *E. coli*- or phage-derived promoter can be used.

Artificially designed and altered promoters such as tac promoter, lacT7 promoter or let I promoter may also be used.

There are no particular limits on the method of introducing the recombinant vector into the bacteria as long as the method can introduce DNA into bacteria, and electroporation or a method employing calcium ions may be used for example.

When a yeast is used for the host cells, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris* or the like may be used. There are no particular limits on the promoter in this case as long as it is capable of expression in yeasts, and for example a gal1 promoter, gal10 promoter, heat shock protein promoter, MFα1 promoter, PH05 promoter, PGK promoter, GAP promoter, ADH promoter, AOX1 promoter or the like can be used.

There are no particular limits on the method of introducing the recombinant vector into the yeast as long as the method can introduce DNA into yeast, and the electroporation, spheroplast or lithium acetate method or the like may be used.

When the host cells are animals cells, monkey COS-7 cells, Vero, chinese hamster ovary cells (CHO cells), mouse L cells, rat GH3, human FL cells or the like may be used as the host cells. There are no particular limits on the

promoter in this case as long as it is capable of expression in animal cells, and for example an SRα promoter, SV40 promoter, LTR (Long Terminal Repeat) promoter, CMV promoter or human cytomegalovirus early gene promoter or the like can be used.

There are no particular limits on the method of introducing the recombinant vector into the animal cells as long as the method can introduce DNA into animal cells, and the electroporation, calcium phosphate or lipofection method or the like can be used.

When the host cells are insect cells, *Spodoptera frugiperda* ovarian cells, *Trichoplusia ni* ovarian cells, cultured cells from silkworm ovaries and the like may be used as the host cells. Possible *Spodoptera frugiperda* ovarian cells include Sf9 and Sf21 cells and the like, *Trichoplusia ni* ovarian cells include High 5 and BTI-TN-5B1-4 (Invitrogen) and the like, and cultured cells derived from silkworm ovaries include *Bombyx mori* N4 cells and the like.

There are no particular limits on the method of introducing the recombinant vector into the insect cells as long as the method can introduce DNA into insect cells, and the calcium phosphate, lipofection or electroporation method or the like can be used.

[Culturing the transformant]

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A transformant into which a recombinant vector with incorporated DNA encoding the target protein has been introduced can be cultured by ordinary culture methods. Culturing of the transformant can be accomplished by ordinary methods used in the culture of host cells.

The medium for culturing a transformant obtained as bacteria, yeasts or other microbial host cells contains carbon sources, nitrogen sources, inorganic salts and the like which can be used by those microorganisms, and either a natural or synthetic medium may be used as long as it is a medium for efficient culturing of the transformant.

Glucose, fructose, sucrose, starch and other carbohydrates, acetic acid, propionic acid and other organic acids, and ethanol, propanol and other alcohols can be used as carbon sources. Ammonia, ammonium chloride, ammonium sulfate, ammonium acetate, ammonium phosphate and other ammonium salts of inorganic and organic acids as well as peptone, meat extract, yeast extract, corn steep liquor, casein hydrolysate and the like can be used as nitrogen sources. Monopotassium phosphate, dipotassium phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate, calcium carbonate and the like can be used as inorganic salts.

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Culturing of a transformant obtained as *E. coli*, yeast or other microbial host cells is accomplished under aerobic conditions such as a shaking culture or aerated spinner culture. The culture temperature is normally 25-37°C, the culture time is normally 12-48 hours, and a pH of 6-8 is maintained during the culture period. The pH can be adjusted using an inorganic acid, organic acid or alkaline solution or urea, calcium carbonate, ammonia or the like. An antibiotic such as ampicillin or tetracycline can be added to the medium as necessary during culture.

When culturing microorganisms transformed with an expression vector employing an inducible promoter as the promoter, an inducer can be added to the medium as necessary. For example, isopropyl-β-D-thiogalactopyranoside or the like can be added to the medium when culturing a microorganism transformed with an expression vector employing a lac promoter, while indoleacrylic acid or the like can be added when culturing a microorganism transformed with an expression vector employing a trp promoter.

Commonly used RPMI1640 medium, Eagle MEM medium, DMEM medium, Ham F12 medium, Ham F12K medium and such medium with fetal calf serum added can be used as the medium for culturing a transformant obtained with animal cells as the host cells. The transformant is normally cultured for 3-10

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days at 37°C under the presence of 5% CO_2. For purposes of culture, an antibiotic such as kanamycin, penicillin, streptomycin or the like can be added to the medium as necessary.

Commonly used TNM-FH medium (Pharmingen), Sf-900 II SFM medium (Gibco BRL), ExCell400 or ExCell405 (JRH Biosciences) or the like can be used as the medium for culturing a transformant obtained with insect cells as the host cells. The transformant is normally cultured for 3-10 days at 27°C. For purposes of culture, an antibiotic such as gentamicin can be added to the medium as necessary.

The target protein can also be expressed as an excreted protein or a fused protein. Examples of proteins to be fused include β-galactosidase, protein A, protein A IgG binding region, chloramphenicol acetyltransferase, poly(Arg), poly(Glu), protein G, maltose binding protein, glutathione S transferase, polyhistidine chain (His-tag), S peptide, DNA binding protein domain, Tac antigen, thioredoxin, and green fluorescent protein.

[Protein isolation and purification]

The target protein is obtained by harvesting the target protein from the transformant culture. "Culture" in this case includes culture supernatant, cultured cells, cultured bacterial bodies, or crushed cells or bacterial cell bodies.

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When the target protein is accumulated in the transformant cells, the cells in the culture are collected by centrifugation of the culture, and the cells are then washed and crushed and the target protein extracted. If the target protein is excreted outside the transformant cells, the culture supernatant is used as is, or else cells or bacterial cell bodies are removed from the culture supernatant by centrifugation or the like.

The resulting protein (a) or (b) can then be purified by a method such as solvent extraction, salting-out desalting with ammonium sulfate or the like, precipitation with organic solvents, diethylaminoethyl (DEAE)-sepharose, ion exchange chromatography, hydrophobic chromatography, gel filtration, affinity chromatography or the like.

Protein (a) or (b) can also be manufactured based on its amino acid sequence by a chemical synthesis method such as the Fmoc (fluorenylmethyloxycarbonyl) or tBoc (t-butyloxycarbonyl) method. A commercial peptide synthesizer can be used in this case.

The antibodies or fragments thereof of the present invention are antibodies or fragments thereof which react to protein (a) or (b). As used here, "antibodies" include both monoclonal and polyclonal antibodies, and "monoclonal and polyclonal antibodies" include all classes of monoclonal and

polyclonal antibodies. "Antibodies" also include antiserum obtained by immunizing rabbits, mice or other immune animals with protein (a) or (b), as well as human antibodies and humanized antibodies obtained by genetic recombination. "Antibody fragments" include Fab fragments, F(ab)'$_2$ fragments, single-chain antibodies (scFv) and the like.

The antibodies or fragments thereof of the present invention are prepared using protein (a) or (b) as the immunizing antigen. For example, (i) crushed or crushed and purified cells or tissue expressing protein (a) or (b), (ii) recombinant protein expressed by introduction of DNA encoding protein (a) or (b) into *E. coli*, insect cells, animal cells or another host by genetic recombination, or (iii) chemically synthesized peptides or the like can be used as the immunizing antigen.

To prepare polyclonal antibodies, rats, mice, guinea pigs, rabbits, sheep, horses, cows or other mammals are immunized with the immunizing antigen. It is preferable to use mice as the immune animals because the antibody can be easily prepared. For immunization, it is preferable from the standpoint of inducing antibody production to use Freund's complete adjuvant or another auxiliary to prepare an emulsion which is then administered multiple times. In addition to Freund's complete adjuvant (FCA), Freund's

incomplete adjuvant (FIA), ammonium hydroxide gel and the like can be used as auxiliaries. The antigen dosage per individual mammal can be determined based on the kind of mammal, but in the case of mice it is ordinarily 50-500 µg. Administration may be intravenous, subcutaneous, intraperitoneal or the like. Immunization is normally performed at intervals of between several days and several weeks, preferably at intervals of 4 days to 3 weeks for a total of 2-8 or preferably 2-5 immunizations. 3-10 days after the last immunization, antibody titer for protein (a) or (b) is measured, and after antibody titer rises blood is taken and antiserum obtained. Antibody titer can be measured by enzyme-linked immunosorbent assay (ELISA), radio-immuno-assay (RIA) or the like.

When antibodies need to be purified from the antiserum, a well known method such as salting out with ammonium sulfate, gel chromatography, ion exchange chromatography, affinity chromatography or the like or a combination of such methods can be selected as appropriate.

To prepare monoclonal antibodies, mammals are immunized using immune antigen as in the case of polyclonal antibodies, and antibody-producing cells are harvested 2-5 days after the final immunization. Examples of antibody-producing cells include spleen cells, lymph node cells, thymus cells,

peripheral blood cells and the like, but spleen cells are normally used.

Next, cell fusion of the antibody-producing cells with myeloma cells is performed to obtain a hybridoma. Commonly available cells strains derived from humans, mice or other mammals can be used as the myeloma cells for fusion with the antibody-producing cells. It is preferable that the cell strain used be one that has drug selectivity and that does not survive in unfused form in selection medium (such as HAT medium), but which only survives when fused with the antibody-producing cells. Specific examples of myeloma cells include P3X63-Ag.8.U1 (P3U1), P3/NSI/1-Ag4-1, Sp2/0-Ag14 and other mouse myeloma cell strains.

Cell fusion is performed by mixing specific proportions (such as between 1:1 and 1:10) of the antibody-producing cells and myeloma cells in an animal cell culture medium such as DMEM or RPMI-1640 which contains no serum, and then performing a fusion reaction with polyethylene glycol or another cell fusion accelerator or by electrical pulse treatment (such as electroporation).

Following cell fusion treatment, culture is performed in a selection medium, and the target hybridoma selected. Next, the culture supernatant of the multiplied hybridoma is screened to confirm the presence of the target antibodies.

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Hybridoma screening may be according to ordinary methods without any particular limitations. For example, part of the culture supernatant in a well containing a hybridoma can be harvested and screened by enzyme-linked immunosorbent assay (ELISA), radio-immuno-assay (RIA) or the like.

The hybridoma can be cloned by limiting dilution analysis, soft agar cloning, fibrin gel cloning, fluorescence activated cell sorting and the like, to finally obtain a hybridoma which produces monoclonal antibodies.

Harvesting of monoclonal antibodies from the resulting hybridoma can be accomplished by an ordinary cell culture method or the like. In the cell culture method, the hybridoma is cultured for example for 3-10 days under ordinary culture conditions (such as 37°C, 5% CO_2) in an animal cell culture medium such as MEM medium or RPMI-1640 medium containing 10-20% fetal calf serum, and monoclonal antibodies are obtained from the culture supernatant. The hybridoma can also be transplanted intraperitoneally to mice or the like, ascites collected after 10-14 days, and monoclonal antibodies obtained from the ascites.

When monoclonal antibodies need to be purified, a well-known method such as salting out with ammonium sulfate, gel chromatography, ion exchange chromatography, affinity

chromatography or the like or a combination of such methods can be selected as appropriate.

When monoclonal antibodies are intended for administration to human beings (antibody therapy), human antibodies or humanized antibodies should be used to reduce immunogenicity. Human antibodies or humanized antibodies are obtained for example by preparing a hybridoma using as the immune animals mice or the like having introduced human antibody DNA, or by using a library having the antibodies presented on a phage. Specifically, a transgenic animal having a repertory of human antibody DNA is immunized with the antigen as a protein or protein-expressing cells or solution thereof to obtain antibody-producing cells, which are then fused with myeloma cells to produce a hybridoma which is used to obtain human antibodies to the target protein (see International Publication Nos. WO92-03918, WO93-2227, WO94-02602, WO96-33735 and WO96-34096). Alternatively, scFv which binds to the target protein can be selected by sorting out, from an antibody library having multiple different human scFv presented on phages, those phages presenting antibodies which bind to the antigen as a protein or protein-expressing cells or solution thereof (Griffiths et al, *EMBO J.* 12, 725-734, 1993).

The screening method of the present invention comprises a step of bringing a test substance into contact with protein (a) or (b). In the screening method of the present invention, a ligand, agonist or antagonist to an adiponectin receptor can be screened by bringing a test substance into contact with protein (a) or (b) and determining whether the test substance binds to protein (a) or (b). The screened substances should also be subjected to another step to determined whether or not they actually act as a ligand, agonist or antagonist to an adiponectin receptor.

There are no particular limits on the types of test substances, but examples include high molecular compounds, low molecular compounds, cell cultures, tissue extracts and the like.

For screening purposes, (i) cells or tissues or processed cells or tissues expressing protein (a) or (b), (ii) recombinant proteins expressed by introduction of DNA encoding protein (a) or (b) into *E. coli*, yeast, insect cells, animal cells or another host by recombinant technology, or (iii) chemically synthesized peptides for example can be used as protein (a) or (b). The cells or tissues expressing protein (a) or (b) may be either cells or tissues (such as muscle cells, skeletal muscles, hearts, macrophages, blood vessels, brains, kidneys, lungs,

placentas, spleens, testes, peripheral blood, thymus glands,

intestines or the like) which express protein (a) or (b) as

an endogenous protein, or cells or tissues which express

protein (a) or (b) as an exogenous protein (such as *E. coli*,

yeast, insect cells, animal cells or the like having

introduced DNA encoding protein (a) or (b)). Processed

cells or tissues are cells are tissues which have been

crushed, extracted, purified or the like, and include for

example cell membrane fractions of cells or tisues.

Whether or not the test substance acts as a ligand,

agonist or antagonist to the adiponectin receptor can be

determined for example based on the amount of binding of the

test substance to protein (a) or (b) or according to the

presence or degree of cell response due to binding of the

test substance to protein (a) or (b).

The amount of binding of the test substance to protein

(a) or (b) can be measured for example using a labeled test

substance, labeled antibodies to the test substance or the

like. Radioactive isotope elements such as ^{3}H, ^{14}C, ^{125}I, ^{35}S,

and ^{32}P and fluorescent dyes and the like can be used as

labels. The radioactivity of the radioactive isotope

element can be measured using a liquid scintillation counter,

X-ray film, imaging plate or the like, while the fluorescent

strength of the fluorescent dye can be measured for example

by a CCD camera, fluorescent scanner, spectrofluorometer or the like.

Examples of cell responses due to binding of the test substance to protein (a) or (b) include stimulation or suppression of PPARα ligand activity, stimulation or suppression of fatty acid oxidation, stimulation or suppression of glucose uptake, increased or decreased intracellular pH, increased or decreased AMP kinase activity, increased or decreased AMP kinase phosphorylation, increased or decreased p38 MAP kinase activity, increased or decreased p38 MAP kinase phosphorylation, stimulation or suppression of gluconeogenesis, and increased or decreased uncoupled protein and the like.

In measuring the amount of binding of the test substance to protein (a) or (b) and the presence or degree of cell response, it is desirable to compare measurement values with and without protein (a) or (b).

Lipid cells secrete adiponectin as a vital insulin sensitizing hormone, and lipid cell hypertrophy leads to decreased adiponectin excretion, which leads to insulin resistance, which in turn is a cause of diabetes, hyperlipidemia and hypertension. Decreased adiponectin excretion also promotes arteriosclerosis. Consequently, substances which have been screened as an agonist or

antagonist to an adiponectin receptor can be used for example as insulin resistance improving drugs, diabetes preventive and therapeutic drugs, hyperlipidemia preventive and therapeutic drugs, hypertension preventive and therapeutic drugs, arteriosclerosis preventive and therapeutic drugs, obesity preventive and therapeutic drugs, anti-inflammatory drugs, osteoporosis preventive and therapeutic drugs, anti-cancer drugs and the like.

These drugs may be composed solely of the screened substances, but normally they are prepared using pharmaceutically acceptable excipients and other additives as desired. For purposes of preparation, excipients, binders, disintegrators, lubricants, stabilizers, flavorings and perfumes, diluents, solvents for injection and other additives can be used for example. Administration may be oral or parenteral (subcutaneous, intramuscular, intraperitoneal or the like) for example, and forms of administration include sprays, capsules, pills, granules, syrups, emulsions, suppositories, injections, suspensions and the like. The dosage and number of administrations will differ according to the desired effects, administration method, treatment period, and age, weight, sex and the like of the patient, and can be adjusted appropriately according to the type of screened substance.

Protein (a) or (b), a gene encoding protein (a) or (b), a recombinant vector containing the DNA and a transformant containing the recombinant vector can be used as constituent elements in a screening kit for a ligand, agonist and antagonist to an adiponectin receptor. They are included in the screening kit as suppliers of protein (a) or (b).

The screening kit can take any form as long as it includes protein (a) or (b), a gene encoding protein (a) or (b), a recombinant vector containing the DNA or a transformant containing the recombinant vector, and can include various reagents (buffers and the like), measurement equipment, labeling compounds, model animals, cell strains, cell culture media and the like.

[Examples]

In the following text and figures relating to the examples, adiponectin may be referred to as "Adipo," adiponectin consisting solely of the globular domain as "globular Adipo" or "gAd," and full-length adiponectin as "full-length Adipo" or "Ad." An adiponectin receptor may be referred to as "AdipoR," a human adiponectin receptor as "hAdipoR," and a mouse adiponectin receptor as "mAdipoR". "AdipoR1" and "AdipoR2" are designations for adiponectin receptors comprising different amino acid sequences.

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1.   Experimental methods

(1)   Retrovirus production and infection

For production of retroviral supernatants, 10^7 Plat-E packaging cells (Morita, S. et al, *Gene Ther.* 7, 1063-1066 (2000)) were transiently transfected with 10 µg of human skeletal muscle cDNA library (Clontech) using Lipofectamine PLUS (Life Technologies). After 24 hours of incubation the supernatants (10 mL) was harvested. Ba/F3 cells were infected with 1/20-diluted supernatants supplemented with 10 µg/mL polybrene (hexadimethrine bromide, Sigma) corresponding to an estimated m.o.i. of 0.3. Six hours later, the medium was changed, and the Ba/F3 cells were expanded by culturing for 6 days prior to freezing or selection.

(2)   FACS analysis and cDNA sequencing

FACS analysis was performed according to the method of Stoecklin et al (Stoecklin, G. et al, *EMBO J.* 21, 4709-4718 (2000)).   For selection, cells were enriched with FACVantage (Becton Dickinson) from 1 x 10^7 Ba/F3 cells transfected with human skeletal muscle cDNA library. The cells were recovered, expanded, and subjected to FACS analysis 11 days later. The selected cells were further

expanded and subjected to FACS analysis. In order to sequence the cDNA incorporated into the selected cells, PCR was performed with upstream and downstream primers for the retrovirus vector, using 50 ng of genome DNA extracted from the selected cells as the template, and the resulting PCR amplified fragments were sequenced.

PCR was performed in 35 cycles of 1 minute at 94°C, 2 minutes at 56°C and 3 minutes at 72°C, using Taq polymerase (Perkin-Elmer/Cetus). The primers (pLIB primers) for the retrovirus vector were as follows:

5' primer:5'- agccctcactccttctctag -3'

3' primer:5'- acctacaggtggggtctttcattccc -3'

After removal of the primer, the base sequence of the PCR product was determined by direct sequencing using a BigDye Terminator Kit (Applied Biosystems).

(3) Northern blot analysis

Human multiple tissues Northern blot filters I and II (brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung, peripheral blood leukocytes) were purchased from Clonetech. These filters were hybridized with [^{32}P] dCTP-labeled cDNA probes (the PstI/BstXI, BamHI/PstI and EcoRV/NotI fragment of human AdipoR1 cDNA, mouse AdipoR1 cDNA, and human and mouse

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AdipoR2 cDNA, respectively) in a hybridization buffer
containing 4 X SSC, 5 X Denhardt's solution, 0.2% SDS, 200
mg/mL salmon sperm DNA and 50% formamide at 42°C for 24
hours. The filters were washed in 0.1 X SSC, 0.1% SDS at
65°C, and then subjected to autoradiography. The same
northern blot analysis was performed on various mouse
tissues (brain, heart, kidney, liver, lung, skeletal muscle,
spleen, testis).

(4) Expression of proteins in mammalian cells, and
characterization

The AdipoR1 or R2 expression vector was constructed by
ligating AdipoR1 or AdipoR2 cDNA into the EcoRV/NotI site of
pCXN2 (Kinoshita, S. et al, *Pharm Res.* 15, 1851-1856 (1998)).
HEK-293T (human embryonic kidney cells), HAEC (normal human
aortic endothelial cells) and C2C12 myocytes (mouse myocyte
strain) were cultured in DMEM. 10% fetal calf serum (FCS)
was contained in the medium. DNA transfection was performed
by lipofection using Lipofectamine PLUS (Gibco BRL) for all
cells.

(5) RNA interference in C2C12 myocytes

According to the method of Karpichev et al (Karpichev,
I.V., *J. Biol. Chem.* 277, 19609-19617 (2002)), two pairs of

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siRNA were chemically synthesized, annealed, and transfected into C2C12 myocytes which had been differentiated into myotube cells by 4-7 days culture in DMEM containing horse serum after 2 days from confluence. siRNA was also transfected into hepatocytes and HAEC in the same way. 48 hours after transfection of siRNA, the cells were lysed.

The base sequences of the unrelated control siRNA (siRNA) are as follows:

Unrelated-sense: gugcgcugcuggugccaacccctt

Unrelated-antisense:ggguuggcaccagcagcgcactt

The base sequences of siRNA (siRNA mAdipoR1) corresponding to the coding region of the mouse AdipoR1 gene are as follows:

siRNA mAdipoR1-sense: gagacuggcaacaucuggacatt

siRNA mAdipoR1-antisense:uguccagauguugccagucuctt

The base sequences of siRNA (siRNA mAdipoR2) corresponding to the coding region of the mouse AdipoR2 gene are as follows:

siRNA mAdipoR2-sense: gcuuagagacaccuguuuguutt

siRNA mAdipoR2-antisense: aacaaacaggugucucuaagctt

The base sequences of siRNA (siRNA hAdipoR1) corresponding to the coding regions of the human AdipoR1 gene are as follows:

siRNA hAdipoR1-sense: ggacaacgacuaucugcuacatt

siRNA hAdipoR1-antisense:uguagcagauagucguugucctt

The base sequences of siRNA (siRNA hAdipoR2) corresponding to the coding regions of the human AdipoR2 gene are as follows:

siRNA hAdipoR2-sense: ggaguuucguuucaugaucggtt

siRNA hAdipoR2-antisense:ccgaucaugaaacgaaacucctt

(6) Measurement of PPARα (Peroxisome proliferator-activated receptor α) ligand activity

Mouse globular Adipo and full-length Adipo expressed using *E. coli* were purified according to the method of Yamauchi et al (Yamauchi, T. et al, *Nat. Med.* 8, 1288-1295 (2002)). According to the method of Yamauchi et al al (Yamauchi, T. et al, *Nat. Med.* 8, 1288-1295 (2002)), the differentiated C2C12 myocytes or isolated hepatocytes were treated with a fixed concentration of adiponectin. PPARα ligand activity was quantitatively determined according to the method of Yamauchi et al (Yamauchi, T. et al, *J. Biol. Chem.* 278, 2461-2468 (2002)) using a (UAS) X 4-tk-LUC reporter plasmid, a GAL4-rat PPARα ligand binding domain expressing plasmid and a β-galactosidase expressing plasmid (internal control).

(7) Lipid and glucose metabolism

[^{14}C] CO_2 production from [$^{1-14}$C] palmitic acid was measured using cell lysate according to the method of Yamauchi et al (Yamauchi, T. et al, *Nat. Med.* 7, 941-946 (2001)). Glucose uptake was also measured according to the method of Yamauchi et al (Yamauchi, T. et al, *Nat. Med.* 8, 1288-1295 (2002)).

(8) Study using dominant negative AMP kinase (AMPK)

cDNA encoding α2 AMPK (including a mutation that alters lysine residue #45 to arginine residue) was used as DN-α2 AMPK (Yamauchi, T. et al, *Nat. Med.* 8, 1288-1295 (2002)). C2C12 myocytes were infected with equal titers of adenovirus containing a control MOCK vector or DN-α2 AMPK. According to the method of Yamauchi et al (Yamauchi, T. et al, *Nat. Med.* 8, 1288-1295 (2002)), 5 days after induction of differentiation, the cells were treated with a fixed concentration of Adipo, and PPARα ligand activity and fatty acid oxidation were measured.

(9) Binding assay

Synthetic human or mouse Adipo was ^{125}I-labeled at Tyr with IODO-beads (Pierce) in the presence of Na^{125}I (2000

Ci/mmol, Amersham Pharmacia Biotech). Recombinant globular Adipo or full-length Adipo was biotinylated with NHS-LC-Biotin (Pierce). The cells were seeded on 96-well plates at a density of 4.1 x 10^4/well. After an overnight culture, the medium was discarded and the cells were incubated overnight at 37°C with a binding assay buffer (HEPES buffered saline/0.1% bovine serum albumin) containing fixed concentrations of [^{125}I] Adipo and unlabeled competitors. According to the methods of Yamauchi et al (Yamauchi, T. et al, *Nat. Med.* 8, 1288-1295 (2002)) ; Yokomizo, T. et al, *Nature* 387, 620-624 (1997)), the cells were then washed three times with ice-cold phosphate-buffered saline and lysed in 0.1N NaOH/0.1% SDS, and the cell-bound radioactivity was determined using a γ-counter.

(10) Fluorescent microscopic analysis

The cellular location of AdipoR1 or AdipoR2 was evaluated by confocal fluorescence microscopy using 293T cells. The cells were fixed in 1% paraformaldehyde, with or without permeabilization using 5 X diluted permeabilization buffer (Coulter), and the cells were incubated for 1 hour at 22°C with anti-FLAG antibody (M2; 30 µg/mL). Then they were incubated with 10 µg/mL of secondary antibody conjugated to AlexFluor 488. Confocal imaging was then performed with a

laser-scanning microscopy system configured with a Nikon microscope and a Krypton/argon laser (488 nm).

(11) Quantitative analysis of AdipoR1 and AdipoR2 gene transcripts by real-time PCR

Quantitative analysis of four transcripts corresponding to the human AdipoR1, human AdipoR2, mouse AdipoR1 and mouse AdipoR2 genes was performed by real-time PCR according to the method of Heid et al (Heid, C.A. et al, *Genome Res.* 6, 986-994 (1996)). The primer sets and the probes for the transcripts were as follows. The PCR product was measured continuously using an ABI PRISM7700 Sequence Detection System (Applied Biosystems). The relative amounts of each transcript were normalized to the amount of actin transcript.

[Mouse AdipoR1 gene]

Forward primer:5'- acgttggagagtcatcccgtat -3'

Reverse primer:5'- ctctgtgtggatgcggaagat -3'

Probe: 5'- cctgctacatggccacagaccacct -3' (with minor groove binder)

[Mouse AdipoR2 gene]

Forward primer:5'- tcccaggaagatgaagggtttat -3'

Reverse primer:5'- ttccattcgttcgatagcatga -3'

Probe: 5'- atgtccccgctcctacaggccc -3' (with minor groove binder)

[Human AdipoR1 gene]

Forward primer:5'- ttcttcctcatggctgtgatgt -3'

Reverse primer:5'- aagaagcgctcaggaattcg -3'

Probe: 5'- tcactggagctggcctttatgctgc -3' (with minor

groove binder)

[Human AdipoR2 gene]

Forward primer:5'- atagggcagataggctggttga -3'

Reverse primer:5'- ggatccgggcagcataca -3'

Probe: 5'- ctgatggccagcctctacatcacagga -3' (with

minor groove binder)

(12) Measurement of intracellular calcium concentration, and

cAMP and cGMP levels

Intracellular Ca^{2+} concentrations were measured

according to the method of Yokomizo et al (Yokomizo, T. et

al, *Nature* 387, 620-624 (1997)). Namely, 10 µM of Fura-2/AM

(Dojin) dissolved in Hepes-Tyrode's BSA buffer (25mM Hepes-

NaOH (pH 7.4), 140 mM NaCl, 2.7 mM KCl, 1.0 mM $CaCl_2$. 12 mM

$NaHCO_3$, 5.6 mM D-glucose, 0.37 mM NaH_2PO_4, 0.49 mM $MgCl_2$,

0.1% [wt/vol] BSA without fatty acids; Fraction V) was

contacted with the cells at 37°C for 2 hours. The cells

were washed twice, and suspended at a concentration of 10^6

cells/mL in Hepes-Tyrode's BSA buffer. 0.5 mL of cell

suspension was applied to a CAF-100 system (Jasco), and 5 µL

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of ligand ethanol solution (for LTB4) or PBS solution (for Adipo) added. Intracellular Ca^{2+} concentrations were measured based on the proportions of 500 nm fluorescence generated by excitation light at 340 and 380 nm.

cAMP and cGMP levels were measured according to the method of Yokomizo et al (Yokomizo, T. et al, *Nature* 387, 620-624 (1997)) using assay kits (Biotrak cAMP EIA System for cAMP, Biotrak cGMP EIA System for cGMP, Amersham Pharmacia Biotech) in accordance with manufacturer's protocols.

(13) Predicted structures of AdipoR1 and AdipoR2

Hydropathy plots of the AdipoR1 and AdipoR2 proteins were conducted using the hydrophobicity indices of Kyte-Doolittle. Structural models for AdipoR1 and AdipoR2 were also predicted by SOSUI, and consensus sequences analyzed by PRINTS (http://bioinf.man.ac.uk/dbbrowser/PRINTS/). In addition, the phosphorylation site was analyzed with DNASIS Pro. Finally, the method described at http://cbrg.inf.ethz.ch/Server/AllAll.html was used to analyze whether AdipoR1/R2 have homology to any other class of GPCR.

(14) Phosphorylation and amount of phosphorylation of AMP kinase (AMPK), ACC, p38 MAP kinase (p38 MAPK) and MAP kinase (MAPK)

Phosphorylation and amount of phosphorylation of AMPK, ACC (Yamauchi, T. et al, *Nat. Med.* 8, 1288-1295 (2002)), p38 MAPK and MAPK (Barger, P.M. et al, *J. Biol. Chem.* 276, 44495-44501 (2001); Puigserver, P. et al, *Mol. Cell* 8, 971-982 (2001); Michael, L.F. et al, *Proc. Natl. Acad. Sci. USA* 98, 3820-3825 (2001)) were measured by western blotting using anti-phosphorylated AMPK antibodies, anti-phosphorylated ACC antibodies, anti-phosphorylated p38 MAPK antibodies and anti-phosphorylated MAPK antibodies. In this test, C2C12 cells or hepatocytes transfected or not transfected with AdipoR1 were incubated for 10 minutes with 0.1 μg/mL or 1 μg/mL gAd, and lysates of the various cells were reacted with their respective antibodies.

2.   Results
(1)   Expression cloning of AdipoR1 and AdipoR2
In muscle, globular Adipo ameliorates insulin resistance and stimulates PPARα and fatty acid oxidation more than does full-length Adipo (Fruebis, J. et al, *Proc. Natl. Acad. Sci. USA* 98, 2005-2010 (2001); Yamauchi, T. et al, *Nat. Med.* 7, 941-946 (2001); Yamauchi, T. et al, *J. Biol.*

Chem. 278, 2461-2468 (2002)). Moreover, globular Adipo binds more strongly to C2C12 cells than does full-length Adipo, and also binds more strongly to skeletal muscle membranes than to hepatocytes and liver membranes (Yamauchi, T. et al, *Nat. Med.* 8, 1288-1295 (2002)) (see Figs. 1a and 1b). Fig. 1a shows binding of globular Adipo or full-length Adipo to C2C12 myocytes, while Fig. 1b shows binding of globular Adipo or full-length Adipo to hepatocytes. The cells were incubated with biotinylated globular Adipo or full-length Adipo at the concentrations shown, and biotinylated globular Adipo or full-length Adipo bound to the cell surfaces was assayed by ELISA. The bars in the figures indicate mean ± s.e. (n = 3-5), with "*" indicating P<0.05 and "**" P<0.01.

An effort was then made to isolate AdipoR1 cDNA by screening proteins with globular Adipo binding ability from a library prepared by infecting Ba/F3 cells with a retrovirus having incorporated cDNA derived from human skeletal muscle mRNA.

The infected Ba/F3 cells were collected, incubated with biotinylated globular Adipo, stained with streptavidin-conjugated phycoerythrin (PE; red fluorescent probe), and subjected to fluorescence-activated cell sorting (FACS) (see Fig. 1c). The results of FACS analysis are shown in Figs.

1c, 1d and 1e. Fig. 1c shows the Ba/F3 cells after staining but before first sorting, Fig. 1d shows the Ba/F3 cells before third sorting, and Fig. 1e shows the Ba/F3 cells after incubation with globular Adipo bound with FITC (fluorescein isothiocyanate) but before fourth sorting. The regions in rectangular boxes in Figs. 1c, 1d and 1e indicate AdipoR1-positive cells, with those in the boxes being selected cells.

The cells selected for globular Adipo binding ability were then subjected to a second round of sorting. The resorted cells that were positive for globular Adipo binding ability (see Fig. 1d) were subjected to a third round of sorting, and the resorted cells immediately incubated with globular Adipo conjugated with FITC (fluorescein isothiocyanate; green fluorescent probe). Since cells which changed from red to green were those having specific binding sites for globular Adipo (see Fig. 1e), only those cells which changed in this way were selected and cultured to expand them for further analysis. Genomic DNA extracted from these cells was subjected to PCR using viral vector primers, and sequenced.

The cells were stained in two colors (red and green) as described above in order to eliminate cells to which

globular Adipo was bound non-specifically by its adhesiveness.

The base sequence of the sequenced human AdipoR1 cDNA is shown by Seq. No. 1, and the amino acid sequence of the protein (human AdipoR1) encoded thereby by Seq. No. 2. The base sequence of mouse AdipoR1 cDNA obtained by similar methods from C2C12 myocytes is shown by Seq. No. 5, and the amino acid sequence of the protein (mouse AdipoR1) encoded thereby by Seq. No. 6. Human AdipoR1 cDNA (Seq. No. 1) and mouse AdipoR1 cDNA (Seq. No. 5) are shown to be genes encoding proteins consisting of 375 amino acids (see Fig. 1f). There is 96.8% homology between the amino acid sequences of human AdipoR1 and mouse AdipoR1 (see Fig. 1f). Fig. 1f shows model structures of AdipoR1 and AdipoR2 gene transcripts in databases (NIH-MGC Project and NCBI contig).

From previous findings (Yamauchi, T. et al, *Nat. Med.* 8, 1288-1295 (2002); Yamauchi, T. et al, *J. Biol. Chem.* 278, 2461-2468 (2002)), it appears that there are two types of AdipoR with distinct binding affinities for globular Adipo or full-length Adipo, one type which preferentially binds globular Adipo expressed in skeletal muscle, and another which binds only full-length Adipo expressed in liver.

A search for proteins that share homology with AdipoR1 led to the discovery in human and mouse databases (The Human

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Genome, http://www.ncbi.nlm.nih.gov/genome/guide/human/;
·Mouse Genome Resources,
http://www.ncbi.nlm.nih.gov/genome/guide/mouse/) (Waterston,
R.H. et al, *Nature* 420, 520-562 (2002); Okazaki, Y. et al,
Nature 420, 563-573 (2002)) of a gene having an open reading
frame (ORF) different from that of AdipoR1 cDNA. This cDNA
was cloned from the mRNA of HepG2 cells (cell strain derived
from human liver cancer) and sequenced, and the protein
encoded by this cDNA was named AdipoR2. The base sequence
of the sequenced human AdipoR2 cDNA is shown by Seq. No. 3,
and the amino acid sequence of the protein encoded thereby
(human AdipoR2) by Seq. No. 4. Furthermore, the base
sequence of mouse AdipoR2 cDNA obtained by the same methods
from C2C12 myocytes is shown by Seq. No. 7, and the amino
acid sequence of the protein encoded thereby (mouse AdipoR2)
by Seq. No. 8.

There is 95.2% homology between the amino acid
sequences of human AdipoR2 and mouse AdipoR2. AdipoR1 and
AdipoR2 are extremely similar structurally, with 66.7%
homology between the amino acid sequences of mouse AdipoR1
and AdipoR2.

In SWISS-PROT, there were no mammalian proteins having
high homology with AdipoR1 and AdipoR2, but interestingly
the cDNA encoding AdipoR1 and AdipoR2 had homology with

yeast YOL002c (Karpichev, I.V. et al, *J. Biol. Chem.* 277, 19609-19617 (2002)). YOL002c is reported to encode a seven transmembrane protein that plays a key role in metabolic pathways which regulate lipid metabolism such as fatty acid oxidation (Karpichev, I.V. et al, *J. Biol. Chem.* 277, 19609-19617 (2002)). It appears that AdipoR/YOL002c with a seven transmembrane structure across species mediates key regulatory signals in lipid metabolism such as fatty acid oxidation.

(2) Tissue distribution of AdipoR1 and AdipoR2

The results of northern blot analysis of various mouse tissues are shown in Fig. 1g, and the results of northern blot analysis of various human tissues in Fig. 1h. In Fig. 1g, lane 1 shows results for the brain, lane 2 for heart, lane 3 for kidney, lane 4 for liver, lane 5 for lung, lane 6 for skeletal muscle, lane 7 for spleen, and lane 8 for testis, while in Fig. 1h, lane 1 shows results for brain, lane 2 for heart, lane 3 for skeletal muscle, lane 4 for colon, lane 5 for thymus, lane 6 for spleen, lane 7 for kidney, lane 8 for liver, lane 9 for small intestine, lane 10 for placenta, lane 11 for lung and lane 12 for peripheral blood leukocytes.

Northern blot analysis of various human and mouse
tissues, identified one major 2.0 kb band with the predicted
mRNA size in the aforementioned database, and also revealed
that AdipoR1 is expressed in most tissues and most
abundantly expressed in skeletal muscle. One major 4.0 kb
band with the predicted mRNA size was also identified in the
aforementioned database, and it was shown that AdipoR2 is
most abundantly expressed in the liver.

(3)   Cellular locations of AdipoR1 and AdipoR2

From the deduced amino acid sequence (Seq. No. 6) of
mouse AdipoR1, it is predicted that mouse AdipoR1 is a
protein consisting of 375 amino acids and having a molecular
weight of 42.4 kDa. From the deduced amino acid sequence
(Seq. No. 8) of mouse AdipoR2, it is predicted that mouse
AdipoR2 is a protein consisting of 311 amino acids and
having a molecular weight of 35.4 kDa (see Fig. 2a). In Fig.
2a, which shows the results of a scan of the AdipoR sequence
by PRINTS software
(http://bioinf.man.ac.uk/dbbrowser/PRINTS/), the underlined
regions are the 7 transmembrane domains of AdipoR1 and
AdipoR2, while the areas underlined in bold indicate the
characteristic conserved motifs of G-protein coupled

receptor members. PKC phosphorylation sites and tyrosine phosphorylation sites are also shown in Fig. 2a.

From the deduced amino acid sequences of AdipoR1 and AdipoR2, it is predicted that AdipoR1 and AdipoR2 are proteins having 7 transmembrane domains (see Fig. 2a). An alignment of AdipoR1 and AdipoR2 was performed with known receptors having 7 transmembrane domains (Waterston, R.H. et al, *Nature* 420, 520-562 (2002); Okazaki, Y. et al, *Nature* 420, 563-573 (2002); Wess, J., *FASE B. J.* 11, 346-354 (1997)), but homology to the amino acid sequences of members of the G-protein coupled receptor (GPCR) family was low. AdipoR1 and AdipoR2 lacked characteristics (such as conserved amino acids, glycosylation sites, sites for G-protein coupling) of the G-protein coupled receptor family (Wess, J. et al, *FASEB. J.* 11, 346-354 (1997); Yokomizo, T. et al, *Nature* 387, 620-624 (1997); Scheer, A. et al, *EMBO. J.* 15, 3566-3578 (1996)). Of the highly conserved amino acids in the G-protein coupled receptor family, only one of two highly conserved Cys residues were present in the first and second extracellular loops of AdipoR1 and AdipoR2. AdipoR1 and R2 lacked the highly conserved Asn-Pro-Xaa2-Tyr motif present at the end of TM7. AdipoR1 and AdipoR2 also lacked the highly conserved Asp-Arg-Tyr motif present at the TM3/intracellular loop 2 transition (Wess, J. et al, *FASEB.*

J. 11, 346-354 (1997); Scheer, A. et al, *EMBO. J.* 15, 3566-3578 (1996)).

Human and mouse AdipoR1 or AdipoR2 labeled with epitope tag FLAG was expressed in HEK-293 cells, and immunoblotted with anti-FLAG antibodies. Human and mouse AdipoR1 and AdipoR2 exhibited the expected molecular weights (see Fig. 2b).

To determine the subcellular location and topology of mouse AdipoR1 and AdipoR2, AdipoR1 or AdipoR2 cDNA with epitope tags at either end was expressed in HEK-293T cells (see Fig. 2c). In Fig. 2c, "intact" indicates that the cells were not permeabilized and "permeabilized" indicates that they were.

When the epitope tag was inserted at the N-terminus, AdipoR1 and AdipoR2 could be detected at the cell surface only when the cells were permeabilized (see Fig. 2c). In contrast, when the epitope tag was inserted at the C-terminus, AdipoR1 and AdipoR2 could be detected at the cell surface (see Fig. 2c). These results indicate that AdipoR1 and AdipoR2 are integral membrane proteins with seven transmembrane domains, in which the N-terminus is within the membrane and the C-terminus is outside the membrane (see Figs. 2c, 2d). This is opposite to the topology of all reported G-protein coupled receptors (Wess, J. et al, *FASEB.*

J. 11, 346-354 (1997); Yokomizo, T. et al, *Nature 381,* 620-624 (1997); Scheer, A. et al, *EMBO J.* 15, 3566-3578 (1996)). Hypothetical structural models of AdipoRl and AdipoR2 are shown in Fig. 2d and 2e, respectively.

(4) Effects of AdipoR expression in 293T cells

The binding and intracellular signaling stimulated by globular Adipo or full-length Adipo using 293T cells overexpressing AdipoRl or AdipoR2 on the cell surface were examined. Expression of AdipoRl or AdipoR2 in 293T cells enhanced binding of both globular Adipo and full-length Adipo (see Figs. 3a, 3b). Fig. 3a shows the binding isotherm of [^{125}I] globular Adipo (gAd) binding to 293T cells transfected with AdipoRl or AdipoR2, while Fig. 3b shows the binding isotherm of [^{125}I] full-length Adipo (Ad) binding to the 293T cells. In these figures, a white square indicates a result for mock, a black square a result for mouse AdipoRl and a black circle a result for mouse AdipoR2.

We next examined whether AdipoRl and AdipoR2 could form multimers. When AdipoRl with the epitope tag FLAG and AdipoRl with the epitope tag HA were co-expressed in HEK-293T cells, AdipoRl with the epitope tag FLAG was detected in anti-HA antibody immunoprecipitates (see Fig. 3c). Fig. 3c shows the results when cell lysates of 293T cells

transfected with AdipoR1 or AdipoR2 having epitope tag FLAG or HA were immunoprecipitated (IP) with anti-FLAG antibody (upper and lower panels), and then immunoblotted with anti-FLAG (upper panel) or anti-HA (lower panel) antibody, and indicates formation of AdipoR1 and AdipoR2 homo- and hetero-multimers.

Moreover, when AdipoR1 with the epitope tag HA and AdipoR2 with the epitope tag FLAG were co-expressed in HEK-293T cells, AdipoR2 with the epitope tag FLAG was detected in anti-HA antibody immunoprecipitates (see Fig. 3c). These data suggest that AdipoR1 and AdipoR2 may be able to form both homo- and hetero-multimers.

In cells expressing AdipoR1, Adipo had no apparent effect on intracellular calcium, although LTB4 increased intracellular calcium in cells expressing either GPCR or LTB4 receptor BLT1 (see Fig. 3d), and these cells showed similar expression levels to those of cells expressing AdipoR1 (data not shown). Fig. 3d shows measurement results for changes in $[Ca^{2+}]i$ when BLT1-expressing cells or mouse AdipoR1-expressing cells loaded with Fura-2/AM were challenged with 10 μg/mL globular Adipo (gAd), 10 μg/mL full-length Adipo (Ad), 1 μM LTB4 or 100 μM ATP.

Moreover, in cells expressing AdipoR1, AdipoR1 had little or no apparent effect on cAMP and cGMP levels (see

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Fig. 3e). Fig. 3e shows results for accumulation of cAMP or cGMP in HEK-293 cells treated with or without forskolin, with "gAd0.01" and "gAd0.1" indicating 0.01 or 0.1 µg/mL of globular Adipo, respectively, and "Ad1" and "Ad10" indicating 1 and 10 µg/mL of full-length Adipo, respectively. In contrast, expression of AdipoR1 enhanced increases in PPARα ligand activity by globular Adipo and full-length Adipo in 293T cells (see Fig. 3f). Fig. 3f shows in PPARα ligand activity in AdipoR1-transfected 293T cells incubated with the indicated concentrations (µg/mL) of globular Adipo or full-length Adipo, with "gAd0.1," "gAd0.5" and "gAd2.5" indicating 0.1, 0.5 and 2.5 µg/mL of globular Adipo, respectively, and "Ad1," "Ad5" and "Ad25" indicating 1, 5 and 25 µg/mL of full-length Adipo, respectively. In the figure each bar shows mean ± s.e. (n = 3-5), with "*" indicating P<0.05 and "**" P<0.01.

(5) PPARα activation and fatty acid oxidation in C2C12 myocytes

Expression of AdipoR1 in C2C12 myocytes (see Fig. 4a) enhanced binding of both globular and full-length Adipo (see Figs 4c, 4d), which was associated with increases in PPARα ligand activity (see Fig. 4e) and fatty acid oxidation (see

Fig. 4f) by globular Adipo and full-length Adipo in C2C12

myocytes. Expression of AdipoR2 in C2C12 myocytes (see Fig.

4b) also enhanced binding of both globular Adipo and full

length-Adipo (see Figs. 4c, 4d), which was associated with

increases in fatty acid oxidation (see Fig. 4f) by globular

and full-length Adipo in C2C12 myocytes. Fig. 4a shows

mouse AdipoR1 mRNA levels and Fig. 4b mouse AdipoR2 mRNA

levels, with results shown using a white bar for Mock, a

black bar for mouse AdipoR1, and a dotted white bar for

mouse AdipoR2. Figs. 4c and 4d show the binding isotherm of

[^{125}I] globular Adipo (gAd) or full-length Adipo (Ad) binding

to C2C12 myocytes transfected with mouse AdipoR1 or mouse

AdipoR2, with a white square indicating Mock, a black square

mouse AdipoR1 and a black circle mouse AdipoR2. Fig. 4e

show PPARα ligand activity in C2C12 myocytes transfected

with mouse AdipoR1 or AdipoR2 when the myocytes were

infected with adenovirus containing LacZ or DN-α2 AMPK and

treated for 7 hours with the indicated concentrations

(μg/mL) of globular Adipo or full-length Adipo, with white

bars indicating results for Mock and black bars for mouse

AdipoR1. Fig. 4f shows in vitro fatty acid oxidation in the

aforementioned C2C12 myocytes, with white bars indicating

results for Mock, black bars for mouse AdipoR1 and dotted

white bars for mouse AdipoR2. In all these figures, the

bars show mean ± s.e. (n = 3-5), with "*" indicating P<0.05
and "**" P<0.01.

Expression of dominant negative AMP kinase did not
affect globular and full-length Adipo-induced and AdipoR1
expression-dependent increases in PPARα ligand activity.
These data strongly suggest that both AdipoR1 and AdipoR2
can mediate binding of globular and full-length Adipo and
stimulate increases in PPARα ligand activity and fatty acid
oxidation by globular and full-length Adipo.

(6) Effects of siRNA on binding and action of AdipoR in
myocytes

To determine whether endogenous AdipoR1 and R2 mediate
the specific binding and metabolic effects of Adipo in
muscle cells, AdipoR1 and R2 expression was suppressed using
siRNA (see Figs. 5a, 5b). Fig. 5a shows mouse AdipoR1 mRNA
levels in C2C12 myocytes transfected with siRNA or mock,
while Fig. 5b shows mouse AdipoR2 mRNA levels in the C2C12
myocytes. In Figs. 5a and 5b, lane 1 shows results using
mock, lane 2 using unrelated siRNA, lane 3 using siRNA for
mouse AdipoR1 gene and lane 4 using siRNA for mouse AdipoR2
gene.

Suppression of AdipoR1 expression by siRNA (see Fig.
5a) in C2C12 myocytes abolished globular Adipo binding

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activity and partially reduced full-length Adipo binding
activity (see Figs. 5c, 5d). Fig. 5c shows results (n = 4)
of a competitive radioligand binding assay in which [^{125}I]
globular Adipo binding to cells transfected with siRNA
duplex was replaced by increasing the concentration of
unlabeled globular Adipo. Fig. 5d shows results (n = 4) of
a competitive radioligand binding assay in which [^{125}I] full-
length Adipo binding to cells transfected with siRNA duplex
was replaced by increasing the concentration of unlabeled
full-length Adipo.

Treatment with either globular or full-length Adipo for
7 hours increased PPARα ligand activity (see Fig. 5g) and
stimulated fatty-acid oxidation (see Fig. 5h) and glucose
uptake (see Fig. 5i) in C2C12 myocytes. Fig. 5g shows
PPARα ligand activity when C2C12 myocytes transfected with
siRNA duplex were incubated for 7 hours with the indicated
concentrations (μg/mL) of globular or full-length Adipo or
with 10^{-5} M Wy-14,643 ("Wy" in the figure). Fig. 5h shows
in vitro fatty acid oxidation when C2C12 myocytes
transfected with siRNA duplex were incubated for 7 hours
with the indicated concentrations (μg/mL) of globular or
full-length Adipo or with 10^{-5} M Wy-14,643 ("Wy" in the
figure). Fig. 5i shows glucose uptake when C2C12 myocytes
transfected with siRNA duplex were incubated for 7 hours

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with the indicated concentrations (μg/mL) of globular or full-length Adipo or with 10^{-7} M insulin ("Ins" in the figure). In Figs. 5g-5i, "gAd0.01," "gAd0.03," "gAd0.1," "gAd0.5" and "gAd2.5" indicate 0.01, 0.03, 0.1, 0.5 and 2.5 μg/mL of globular Adipo, respectively, while "Ad0.1," "Ad0.3," "Ad1," "Ad5" and "Ad25" indicate 0.1, 0.3, 1, 5 and 25 μg/mL of full-length Adipo, respectively. In the figures, each bar shows mean ± s.e. (n = 3-5), with "*" indicating $P<0.05$ and "**" $P<0.01$. In Figs. 5g-5i, white bars indicate results using unrelated siRNA, black bars using siRNA for mouse AdipoR1, dotted white bars using siRNA for mouse AdipoR2, and shaded bars using siRNA for mouse AdipoR1 and mouse AdipoR2.

Suppression of AdipoR1 expression by siRNA in C2C12 myocytes (see Fig. 5a) reduced increases in PPARα ligand activity (see Fig. 5g), fatty acid oxidation (see Fig. 5h) and glucose uptake (see Fig. 5i) by globular Adipo. In contrast, suppression of AdipoR1 expression failed to significantly reduce these effects by full-length Adipo. Thus, AdipoR1 appears to mediate increases in PPARα ligand activity, fatty acid oxidation and glucose uptake by globular Adipo in muscle cells.

To determine whether endogenous AdipoR2 mediates the specific binding and metabolic effects of Adipo in muscle

cells, AdipoR2 expression was suppressed using siRNA (see Fig. 5b). Suppression of AdipoR2 expression by siRNA in C2C12 myocytes (see Fig. 5b) partially reduced both globular and full-length Adipo binding (see Figs. 5e, 5f). Moreover, suppression of AdipoR2 expression partially reduced increases in PPARα ligand activity (see Fig. 5g) and fatty acid oxidation (see Fig. 5h) by full-length Adipo. Thus, AdipoR2 appears to partially mediate increases in PPARα ligand activity and fatty acid oxidation by full-length Adipo in muscle cells. Fig. 5e shows the binding isotherm of [^{125}I] globular Adipo binding to C2C12 myocytes transfected with siRNA duplex, and Fig. 5f shows the binding isotherm of [^{125}I] full-length Adipo binding to C2C12 myocytes transfected with siRNA duplex. In the figures, white squares indicate results using unrelated control siRNA, black circles using siRNA for mouse AdipoR2, and black triangles using siRNA for mouse AdipoR1 and mouse AdipoR2.

It appears that AdipoR1 is a receptor with relative selectivity for globular Adipo, and AdipoR2 is a receptor with relative selectivity for full-length Adipo. However, suppressing the functional expression of either type of AdipoR has significant effects on both globular and full-length Adipo. These results may be explained by the

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observation that AdipoR1 and AdipoR2 may form both homo- and hetero-multimers (see Fig. 3e).

Interestingly, simultaneous suppression of AdipoR1 and AdipoR2 expression with siRNA in C2C12 myocytes almost abolished both globular Adipo and full-length Adipo binding (see Fig. 5e, 5f), and increases in PPARα ligand activity (see Fig. 5g) and fatty acid oxidation (see Fig. 5h) by globular and full-length Adipo.

(7)   Effects of siRNA on binding and action in hepatocytes

The binding of Adipo to hepatocytes was studied. Hepatocytes showed specific binding for full-length Adipo (see Fig. 6b). Expression of AdipoR1 and R2 enhanced binding of globular and full-length Adipo to hepatocytes (see Figs. 6a, 6b). Conversely, suppression of AdipoR2 expression by siRNA in hepatocytes greatly reduced full-length Adipo binding (see Fig. 6b). These data appear to suggest that AdipoR1 is a receptor with relative selectivity for globular Adipo, while AdipoR2 is a receptor with relative selectivity for full-length Adipo.

The binding of Adipo to Normal Human Aortic Endothelial Cells (HAEC) was studied. Simultaneous suppression of both AdipoR1 and AdipoR2 expression with siRNA greatly reduced globular Adipo binding (see Fig. 6e), and partially reduced

full-length Adipo binding (see Fig. 6f). These data indicate that AdipoR1 and AdipoR2 are also receptors for Adipo in HAEC.

(8) Scatchard plot analysis

Figs. 7a and 7b show the results of the tests of Figs. 5e and 5f conducted again in more detail. Fig. 7a (which corresponds to Fig. 5e) shows the binding isotherm of [^{125}I] globular Adipo binding to C2C12 myocytes transfected with siRNA duplex , while Fig. 7b (which corresponds to Fig. 5f) shows the binding isotherm of [^{125}I] full-length Adipo binding to C2C12 myocytes transfected with siRNA duplex. In Figs. 7a and 7b, white squares indicate results using unrelated control siRNA, black squares using siRNA for mouse AdipoR1, black circles using siRNA for mouse AdipoR2, and black triangles using siRNA for mouse AdipoR1 and mouse AdipoR2.

Scatchard plot analysis was carried out based on the results shown in Figs. 7a and 7b, with the results shown in Figs. 7c and 7d.

C2C12 myocytes transfected with unrelated siRNA bound more strongly to globular Adipo than to full-length Adipo (see Figs. 7a and 7b).

Scatchard plot analysis revealed that there are two types of binding sites for globular Adipo ; high affinity binding sites (Kd value about 0.06 µg/mL, equivalent to 1.14 nM of gAd trimer) and intermediate affinity binding sites (Kd value about 0.80 µg/mL, equivalent to 14.4 nM of gAd trimer) (see Fig. 7c), and there are also two types of binding sites for full-length Adipo ; intermediate affinity binding sites (Kd value about 6.7 µg/mL, equivalent to 49.1 nM of Ad hexamer) and low affinity binding sites (Kd value about 329.3 µg/mL, equivalent to 2415 nM of Ad hexamer) (see Fig. 7d).

Suppression of AdipoR1 expression with siRNA largely reduced globular Adipo binding (see Fig. 7a), but only barely reduced full-length Adipo binding (see Fig. 7b). Scatchard plot analysis revealed that specific suppression of AdipoR1 abrogated high affinity binding sites for globular Adipo, but failed to affect intermediate affinity binding sites for globular Adipo (see Fig. 7c). Moreover, Scatchard plot analysis revealed that specific suppression of AdipoR1 expression only partially reduced the activity of intermediate affinity binding sites for full-length Adipo, but abrogated low affinity binding sites for full-length Adipo (see Fig. 7d).

By contrast with AdipoR1, suppression of AdipoR2 expression with siRNA largely reduced full-length Adipo binding (see Fig. 7b), but barely reduced globular Adipo binding (see Fig. 7a). Scatchard plot analysis revealed that specific suppression of AdipoR2 only partly reduced the activity of high affinity binding sites for globular Adipo, but abrogated intermediate affinity binding sites for globular Adipo (see Fig. 7c). Moreover, Scatchard plot analysis revealed that specific suppression of AdipoR2 abrogated intermediate affinity binding sites for full-length Adipo, but failed to affect the activity of low affinity binding sites for full-length Adipo (see Fig. 7d).

(9) Phosphorylation and amount of phosphorylation of AMPK, ACC, p38 MAPK and MAPK

The panels in Fig. 8a show the results when C2C12 cells transfected ("AdipoR1" in figures) or not transfected ("Mock" in figures) with AdipoR1 were incubated for 10 minutes with 0.1 or 1 μg/mL of gAd, and a lysate of those cells was then reacted with anti-phosphorylated AMPK antibodies, while the panels in Fig. 8b show the results when a lysate of those cells was reacted with anti-phosphorylated ACC antibodies, the panels in Fig. 8c show the results when a lysate of those cells was reacted with

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anti-phosphorylated p38 MAPK antibodies, and the panels in

Fig. 8d show the results when a lysate of those cells was

reacted with anti-phosphorylated MAPK antibodies. The

panels in Fig. 8e show the results when hepatocytes

transfected ("AdipoR1" in figures) or not transfected

("Mock" in figures) with AdipoR1 were incubated for 10

minutes with 0.1 or 1 μg/mL of gAd and a lysate of those

cells was then reacted with anti-phosphorylated AMPK

antibodies, while the panels in Fig. 8f show the results

when a lysate of those cells was reacted with anti-

phosphorylated ACC antibodies. The graphs below the panels

in Figs. 8a-8f show amounts of phosphorylation at each

position on the panel. In the figures, "pAMPK" is

phosphorylated AMPK, "pACC" is phosphorylated ACC, "pp38

MAPK" is phosphorylated p38 MAPK, "pp44 MAPK is

phosphorylated p44MAPK, and "pp42 MAPK" is phosphorylated

p42 MAPK.

In C2C12 myocytes not transfected with AdipoR1, both

globular Adipo and full-length Adipo increased amounts of

phosphorylation of AMPK, ACC and p38 MAPK, but did not

increase phosphorylation of MAPK and other protein kinases

(see Figs. 8a-8d). Expression of AdipoR1 in C2C12 cells was

related to stimulation of phosphorylation of AMPK, ACC and

p38 MAPK by globular Adipo (see Figs. 8a-8d). This suggests

that AdipoR1 mediates activation of AMPK and p38 MAPK by globular Adipo.

In hepatocytes not transfected with AdipoR1, full-length Adipo promoted AMPK activation and ACC phosphorylation, while globular Adipo did not (see Figs. 8e and 8f). Expression of AdipoR1 in hepatocytes was related to stimulation of AMPK and ACC phosphorylation by globular and full-length Adipo (see Figs. 8e and 8f). This suggests that AdipoR1 mediates AMPK and ACC phosphorylation by globular and full-length Adipo.

In C2C12 myocytes not transfected with AdipoR1 ("Mock" in Fig. 8), the fatty acid oxidation and glucose uptake stimulated by globular Adipo were partially inhibited by dominant negative (DN) AMPK or the p38 MAPK specific inhibitor SB203580 (Barger, P.M. et al, *J. Biol. Chem.* 276, 44495-44501 (2001); Puigserver, P. et al, *Mol. Cell* 8, 971-982 (2001); Michael, L.F. et al, *Proc. Natl. Acad. Sci. USA* 98, 3820-3825 (2001)) (see Figs. 8g and 8h). Expression of AdipoR1 in C2C12 myocytes ("mAdipoR1" in Fig. 8) promoted fatty acid oxidation and glucose uptake by globular Adipo, but this effect was also partially inhibited by DN-AMPK or SB203580 (see Figs. 8g and 8h). Thus, the stimulation of fatty acid oxidation and glucose uptake by globular Adipo

via AdipoR1 appeared to be associated with both AMPK and p38 MAPK pathways in C2C12 myocytes.

From the results above, it appears that the AdipoR1 cDNA (Seq. Nos. 1 & 5) and AdipoR2 cDNA (Seq. Nos. 3 & 7) obtained in these examples encodes AdipoR1 (Seq. Nos. 2 & 6) and AdipoR2 (Seq. Nos. 4 & 8) having biological functions.

INDUSTRIAL APPLICABILITY

The present invention provides a novel protein having adiponectin binding ability, a gene encoding the aforementioned protein, a recombinant vector containing the aforementioned gene, a transformant containing the aforementioned recombinant vector and and antibody to the proteins. Moreover, the present invention provides a screening method and screening kit for screening a ligand, agonist and antagonist to an adiponectin receptor using the aforementioned protein, gene, recombinant vector or transformant.